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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/19, 15/24, C12P 21/02 A61K 37/02, C07K 13/00, 3/28 C12N 5/16, 1/21	A1	(11) International Publication Number: WO 92/05256 (43) International Publication Date: 2 April 1992 (02.04.92)
(21) International Application Number: PCT/US91/06332 (22) International Filing Date: 4 September 1991 (04.09.91) (30) Priority data: 584,941 18 September 1990 (18.09.90) US (71) Applicants: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). THE WISTAR INSTITUTE [US/US]; 36th and Spruce Streets, Philadelphia, PA 19104 (US). (72) Inventors: TRINCHIERI, Giorgio ; 355 Wister Road, Wynnewood, PA 19104 (US). PERUSSIA, Bice ; 2302 Waverly Street, Philadelphia, PA 19146 (US). KOBAYASHI, Michiko ; 175 Freeman Street, Apartment 404, Brookline, MA 02146 (US). CLARK, Steven, C. ; 122 Johnson Road, Winchester, MA 01890 (US). WONG, Gordon, G. ; 40 Jamaica Way, Apartment 10, Jamaica Plain, MA 02130 (US). HEWICK, Rodney ; 16 Woodcliffe Road, Lexington, MA 02173 (US).		(74) Agent: KAPINOS, Ellen, J.; Genetics Institute, Inc., 87 CambridgePark Drive, Inc., Cambridge, MA 02140 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: NATURAL KILLER STIMULATORY FACTOR (57) Abstract A novel homogeneous human cytokine, natural killer stimulatory factor, having the ability to induce the production of gamma interferon <i>in vitro</i> in human peripheral blood lymphocytes, and a pharmaceutical preparation containing it.		

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NATURAL KILLER STIMULATORY FACTOR

This is a continuation-in-part of pending U. S. patent application Serial Number 07/307,817, filed on
5 February 7, 1989, which is a continuation-in-part of U.S. patent application Serial Number 07/269,945, filed November 10, 1988.

The present invention relates to a novel cytokine that stimulates the function of natural killer
10 cells and other cells of the immune system, and to processes for obtaining the factor in homogeneous form and producing it by recombinant genetic engineering techniques.

Background of the Invention

15 Natural killer (NK) cells are a subset of lymphocytes active in the immune system and representing an average 15% of mononuclear cells in human peripheral blood [G. Trinchieri and B. Perussia, Lab. Invest.,

50:489 (1984)]. Among the surface markers used to identify human NK cells is a receptor binding with low affinity to the Fc fragment of IgG antibodies, such as Fc-gamma receptor III or CD16 antigen [B. Perussia et al, 5 J. Immunol., 133:180 (1984)]. NK cells have been demonstrated to play an important role in vivo in the defense against tumors, tumor metastases, virus infection, and to regulate normal and malignant hematopoiesis.

10 A growing family of regulatory proteins that deliver signals between cells of the immune system has been identified. These regulatory molecules are known as cytokines. Many of the cytokines have been found to control the growth, development and biological activities 15 of cells of the hematopoietic and immune systems. These regulatory molecules include all of the colony-stimulating factors (GM-CSF, G-CSF, M-CSF, and multi CSF or interleukin-3), the interleukins (IL-1 through IL-11), the interferons (alpha, beta and gamma), the tumor 20 necrosis factors (alpha and beta) and leukemia inhibitory factor (LIF). These cytokines exhibit a wide range of biologic activities with target cells from bone marrow, peripheral blood, fetal liver, and other lymphoid or hematopoietic organs. See, e.g., G. Wong and S. Clark, 25 Immunology Today, 9(5):137 (1988).

The biochemical and biological identification and characterization of certain cytokines was hampered by the small quantities of the naturally occurring factors available from natural sources, e.g., blood and urine.

5 Many of the cytokines have recently been molecularly cloned, heterologously expressed and purified to homogeneity. [D. Metcalf, "The Molecular Biology and Functions of the Granulocyte-Macrophage Colony Stimulating Factors," Blood, 67(2):257-267 (1986).]

10 Among these cytokines are gamma interferon, human and murine GM-CSF, human G-CSF, human CSF-1 and human and murine IL-3. Several of these purified factors have been found to demonstrate regulatory effects on the hematopoietic and immune systems in vivo, including GM-

15 CSF, G-CSF, IL-3 and IL-2.

There remains a need in the art for additional proteins purified from their natural sources or otherwise produced in homogeneous form, which are capable of stimulating or enhancing immune responsiveness and are

20 suitable for pharmaceutical use.

Brief Summary of the Invention

In one aspect the present invention provides a novel human natural killer stimulatory factor, called NKSF, which is substantially free from other mammalian

25 proteins. Active NKSF has an apparent molecular weight

of approximately 70-80 kD. Pure preparations of NKSF reveal the presence of two polypeptides, subunits of approximately 40 kD and 30kD, which, when associated, yield active NKSF. It is presently speculated that NKSF is a heterodimer formed by association of both the larger and smaller subunits through one or more disulfide bonds. This apparent heterodimeric structure can be generated by association of the two individual subunits.

The active, approximately 70-80 kD, NKSF is further characterized by containing all or a portion of the amino acid sequences of Table I and/or II below. Additionally, one or more of nine sequences of amino acids is present in the primary sequence of either the larger or smaller of the NKSF subunits. These nine amino acid fragments are listed and discussed in detail below.

The larger subunit polypeptide of NKSF is characterized by having an apparent molecular weight of 40kD. This subunit is further characterized by having the same or substantially the same amino acid sequence as described in Table I, containing the N-terminal sequence:

Ile-Trp-Glu-Leu-Lys-Lys-Asp-Val-Tyr-Val-Val-Glu-Leu-Asp-Trp-Tyr-Pro-Asp-Ala-Pro-Gly-Glu-Met. This N-terminal amino acid sequence corresponds to amino acids #

23 - 45 of Table I. This polypeptide is further characterized by containing six of the nine amino acid fragments.

5 The smaller polypeptide subunit of NKSF is characterized by an apparent molecular weight of approximately 30-35 kD. Two cDNA sequences have been identified for the smaller subunit. The shorter of the two sequences is substantially contained within the longer sequence in plasmid p35nksf14-1-1, illustrated in
10 Table II. The smaller subunit is further characterized by having the same or substantially the same amino acid sequence as described in Table II, containing the following N-terminal sequence:

Arg-Asn-Leu-Pro-Val-Ala-Thr-Pro-Asp-Pro-Gly-
15 Met-Phe-Pro. This fragment corresponds to underlined amino acids #57-70 of the p35nksf14-1-1 clone.

This smaller polypeptide is further characterized by containing three of the nine fragments of amino acids identified by underlining in Table II.

20 NKSF displays biological activity in inducing the production of gamma interferon in vitro in human peripheral blood lymphocytes (PBLs). In homogeneous form, NKSF is characterized by a specific activity of greater than 1×10^7 dilution units per milligram in the gamma
25 interferon induction assay, described in detail below.

In addition to the induction of gamma interferon in PBLs, NKSF demonstrates the following biological activities:

- (1) biological activity in a granulocyte-macrophage colony stimulating factor (GM-CSF) inducing assay with PBLs;
- (2) biological activity in activating Natural Killer (NK) cells to kill leukemia and tumor-derived cells;
- (3) biological activity in a tumor necrosis factor (TNF) inducing assay with phytohemagglutinin (PHA)-activated T lymphocytes;
- (4) co-mitogenic activity with peripheral blood T lymphocytes; and
- (5) synergizes with IL-2 in inducing γ IFN production in PBLs and maintaining PBL proliferation.

Another aspect of the invention includes DNA sequences comprising cDNA sequences encoding the expression of a human NKSF polypeptide, a human NKSF larger subunit polypeptide, and a human NKSF smaller subunit polypeptide. Such sequences include a sequence of nucleotides encoding one or more of the subunits and peptide sequences described above.

Also provided by the present invention is a vector containing a DNA sequence encoding NKSF or a subunit of NKSF in operative association with an

expression control sequence. Host cells transformed with such vectors for use in producing recombinant NKSF or its recombinant subunits are also provided by the present invention.

5 As still a further aspect of the present invention, there is provided recombinant NKSF protein. This protein is free from other mammalian proteinaceous materials and is characterized by the presence of a DNA sequence encoding one or more of the above-described
10 subunits or peptide fragments containing one or more of the above-described physical, biochemical or biological activities or characteristics.

 Another aspect of this invention provides pharmaceutical compositions containing a therapeutically
15 effective amount of homogeneous or recombinant NKSF, or an effective amount of one or both of the subunits of NKSF, or of one or more of the peptide fragments thereof. These pharmaceutical compositions may be employed in methods for treating cancer, viral infections, such as
20 AIDS, bacterial infections, and other disease states responsive to the enhanced presence of gamma interferon or GM-CSF production. Thus, generally this factor may be employed in the treatment of diseases in which stimulation of immune function might be beneficial.

A further aspect of the invention, therefore, is a method for treating cancer and/or other pathological states which may benefit from enhanced natural killer cell functions by administering to a patient a
5 therapeutically effective amount of NKSF or one or both of its subunits or peptide fragments thereof in a suitable pharmaceutical carrier. These therapeutic methods may include administering simultaneously or sequentially with NKSF or one or more of its subunits or
10 peptide fragments an effective amount of at least one other cytokine, hematopoietin, interleukin, growth factor, or antibody. Specifically, the administration of NKSF or one or more of its subunits with IL-2 has demonstrated synergistic effects. Because of the synergy
15 with IL-2 in vitro, this interleukin might be particularly effective in combination with NKSF.

Still a further aspect of the present invention is a process for producing homogeneous NKSF, or a subunit thereof from a human cell line producing NKSF or a
20 subunit thereof in admixture with other proteins and polypeptides. This process of production provided by the present invention includes culturing selected cells capable of producing NKSF, its subunits, or peptide fragments thereof to obtain conditioned medium and
25 purifying the conditioned medium through five primary purification steps.

The vectors and transformed cells of the invention are employed in another aspect, a novel process for producing recombinant human NKSF protein, a subunit thereof or peptide fragments thereof. In this process a
5 cell line transformed with a DNA sequence encoding on expression NKSF protein, a subunit thereof or a peptide fragment thereof in operative association with an expression control sequence therefore is cultured. This claimed process may employ a number of known cells as
10 host cells for expression of the polypeptide. Presently preferred cell lines are mammalian cell lines and bacterial cells.

Other aspects and advantages of the present invention will be apparent upon consideration of the
15 following detailed description of preferred embodiments thereof.

Detailed Description of the Invention

The novel human natural killer cell stimulatory factor, NKSF, provided by the present invention is a
20 homogeneous protein or proteinaceous composition substantially free of association with other mammalian proteinaceous materials.

Natural killer stimulatory factor has an apparent molecular weight of approximately 70-80 kD as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions.

5 This 70-80 kD peptide is active in a gamma interferon induction assay.

Under reducing conditions in SDS-PAGE, the 70-80 kD band yields two smaller subunits with apparent molecular weights of approximately 40 kD (larger subunit) and approximately 30-35 kD (smaller subunit). For both
10 subunits individually, the biological activity in the same gamma interferon induction assay is substantially lost compared to that of the native 70-80 kD species. The amino terminal sequences identified above were
15 originally determined from the 40 kD reduced species and the 30-35 kD reduced species believed to be the subunits of the NKSF heterodimer. It is presently believed that NKSF is a disulfide-bonded heterodimer of the larger and smaller subunits. However, it is also possible that one
20 or both of these subunits, when present alone, may have biological activity.

NKSF is, at least in part, an anionic glycoprotein. Under isoelectric focusing, two species of the NKSF are observed having isoelectric points of 4.3
25 and 4.8. It is presently speculated that the two species differ in glycosylation patterns.

NKSF is primarily characterized by biological activity in the gamma interferon induction assay described in detail in Example 8 below. Among its other biological activities include the ability to induce GM-CSF production by human peripheral blood lymphocytes. [See, e.g., published PCT application W086/00639 for additional information on GM-CSF]. NKSF also has an enhancing effect on the mitogenic activity of various mitogens, such as lectins and phorbol diesters, on peripheral blood T lymphocytes and has a growth promoting effect on activated human tonsillar B cells. NKSF has also been observed to enhance NK cell functions to kill leukemia and tumor-derived cells in vitro using a spontaneous cell cytotoxicity assay and an antibody dependent cell cytotoxicity (ADCC) assay.

In a spontaneous cell cytotoxicity assay, human peripheral blood lymphocytes or purified NK cells are incubated in the presence of NKSF for a period of 8 to 18 hours. Lymphocytes and NK cells are then assayed in a standard ⁵¹Cr-release assay for their ability to lyse target cells such as leukemia cell lines, tumor-derived cell lines, or virus-infected fibroblasts. NKSF dramatically increases the ability of NK cells to lyse such target cells at a level comparable to that obtained with interferon alpha and IL-2, well known activators of

NK cell cytotoxic activity [See, e.g., G. Trinchieri et al, J. Exp. Med., 147:1314 (1978) and G. Trinchieri et al, J. Exp. Med., 160:1146 (1984)].

5 In an ADCC assay target cancer cells are coated with antibodies capable of binding to the Fc receptor on NK cells, e.g., IgG₂, IgG₃ and the like. In preliminary assays, the presence of NKSF appears to enhance the killing activity of the NK cells for the coated tumor cells in ADCC. [See, e.g., L. M. Weiner et al, Cancer
10 Res., 48:2568-2573 (1988); P. Hersey et al, Cancer Res., 46:6083-6090 (1988); and C. J. Hansik et al, Proc. Natl. Acad. Sci., 83:7893-97 (1986) for additional information on ADCC.]

Preliminary analysis of NKSF in a B-cell growth
15 factor assay using normal human B cells stimulated with goat anti-human IgM antibody (anti- μ) coupled to beads indicates that NKSF may also be characterized by B cell growth factor activity. In this assay the antibody directed against the IgM immunoglobulin on the surface of
20 the B cell activates the B cell and causes it to become responsive to B cell growth factors. [See, C-T K. Tseng et al, J. Immunol., 140:2305-2311 (1988)]. Such antibodies are commercially available.

NKSF was originally detected in the conditioned medium of the human cell line, RPMI 8866, a commercially available cell line [University of Pennsylvania Cell Center] which produces a mixture of lymphokines. This factor may also be produced by other Epstein Barr virus-transformed lymphoblastoid cell lines or from other human cell lines. The RPMI 8866 cell line produces the factor spontaneously, but the level of production can be enhanced by treating the cell line with phorbol esters, such as phorbol dibutyrate. The cells deprived of serum for 48 hours still produce NKSF along with other lymphokines. Procedures for culturing RPMI 8866 (see Example 1) or another cell source of NKSF are known to those of skill in the art.

The purification technique employed in obtaining NKSF from cells which naturally produce it, uses the following steps. These steps include purification through an ion exchange column, e.g., QAE Zeta preparative cartridge [LKB Pharmacia], which indicates that the NKSF protein is anionic. The second purification step is a lentil lectin column which demonstrates that NKSF is, at least in part, a glycoprotein. The eluate from the lentil lectin column is further purified through a hydroxylapatite column, followed by a heparin sepharose column and a fast protein

liquid chromatography (FPLC) Mono-Q column. The NKSF from RPMI 8866 eluted as a single peak in each of the three latter columns. A remaining protein contaminant of about 37 kD is removed by gel filtration chromatography alone or reverse phase HPLC and gel filtration chromatography. The resulting purified homogeneous NKSF was assayed for biological activity in the gamma interferon induction assay of Example 8 and demonstrated a specific activity of greater than 1×10^7 dilution units per milligram.

Thus, the homogeneous NKSF may be obtained by applying the above purification procedures, which are described in detail in Example 2 to the conditioned medium of RPMI 8866 or other sources of human NKSF.

NKSF, one or both of its subunits, or peptide fragments thereof may also be produced via recombinant techniques, e.g., by culturing under suitable conditions a host cell transfected with DNA sequences encoding the larger and/or smaller subunit in operative association with a regulatory control sequence capable of directing expression thereof.

The DNA sequences for cloned NKSF and its subunits were originally isolated by preparing tryptic digests of the homogeneous polypeptide. For example, the nine tryptic fragments originally found in NKSF are identified below:

15

Fragment 1: Leu-Thr-Ile-Gln-Val

Fragment 2: Lys-Tyr-Glu-Asn-Tyr-Thr

Fragment 3: Ile-Trp-Glu-Leu-Lys

Fragment 4: Leu-Met-Asp-Pro-Lys

5 Fragment 5: Val-Met-Ser-Tyr-Leu-Asn-Ala

Fragment 6: Ala-Val-Ser-Asn-Met-Leu-Gln-Lys

Fragment 7: Asn-Ala-Ser-Ile-Ser-Val

Fragment 8: Thr-Phe-Leu-Arg

Fragment 9: Asp-Ile-Ile-Lys-Pro-Asp-Pro-Pro-Lys.

10 Fragments 4, 5 and 6 have been identified as being
located within the smaller or 30 kD subunit. These
sequences correspond to the underlined amino acids #179-
184, 246-252, and 81-86, respectively, of the p35nksf14-
1-1 clone illustrated in Table II. Fragments 1-3 and 7-9
15 have been identified as being located within the larger,
40 kD, NKSF subunit. Amino acid sequences corresponding
to Fragment 1 (amino acids #75-79); Fragment 2 (amino
acids #219-224); Fragment 3 (amino acids #23-27);
Fragment 7 (amino acids #303-308); Fragment 8 (amino
20 acids #127-130); and Fragment 9 (amino acids #231-239)
are underlined in Table I. Additionally, the amino
terminal sequences of the larger and smaller subunits of
NKSF were identified as described below in Example 5 and
are underlined in Table I (#23-45) and Table II (#57-70),
25 respectively.

Oligonucleotide probes were synthesized using the genetic code to predict all possible sequences that encode the amino acid sequences of these tryptic digestion products of NKSF. The same procedure may be followed by constructing probes from the above-identified amino terminal sequences of the two subunits of NKSF. The NKSF subunit genes can be identified by using these probes to screen a human genomic library. Alternatively, the mRNA from RPMI 8866 or another cell source of NKSF can be used to make a cDNA library which can be screened with the probes to identify the cDNAs encoding the polypeptides of the NKSF large and small subunits. Once the cDNAs were identified, they were introduced into an expression vector to make an expression system for NKSF, or one or both of its subunits.

By such use of recombinant techniques, DNA sequences encoding the polypeptides of the NKSF large and small subunit were obtained, which contain DNA sequences encoding the tryptic fragments or the amino terminal sequences identified above.

One NKSF clone, named pNK40-4, has the DNA and amino acid sequences presented in Table I below and codes for all or a portion of the larger NKSF subunit:

17

TABLE I

pNK40-4
cDNA nucleotide and amino acid sequence,
40 kD subunit of NKSF

5	GAATTCCGTC GACTCTAGAG GCCCAGAGCA AG ATG TGT CAC CAG	44
	Met Cys His Gln	
	1	
10	CAG TTG GTC ATC TCT TGG TTT TCC CTG GTT TTT CTG GCA	83
	Gln Leu Val Ile Ser Trp Phe Ser Leu Val Phe Leu Ala	
	5 10 15	
15	TCT CCC CTC GTG GCC ATA TGG GAA CTG AAG AAA GAT GTT	122
	Ser Pro Leu Val Ala <u>Ile Trp Glu Leu Lys Lys Asp Val</u>	
	20 25 30	
20	TAT GTC GTA GAA TTG GAT TGG TAT CCG GAT GCC CCT GGA	161
	<u>Tyr Val Val Glu Leu Asp Trp Tyr Pro Asp Ala Pro Gly</u>	
	35 40	
25	GAA ATG GTG GTC CTC ACC TGT GAC ACC CCT GAA GAA GAT	200
	<u>Glu Met</u> Val Val Leu Thr Cys Asp Thr Pro Glu Glu Asp	
	45 50 55	
30	GGT ATC ACC TGG ACC TTG GAC CAG AGC AGT GAG GTC TTA	239
	Gly Ile Thr Trp Thr Leu Asp Gln Ser Ser Glu Val Leu	
	60 65	
35	GGC TCT GGC AAA ACC CTG ACC ATC CAA GTC AAA GAG TTT	278
	Gly Ser Gly Lys Thr <u>Leu Thr Ile Gln Val</u> Lys Glu Phe	
	70 75 80	
40	GGA GAT GCT GGC CAG TAC ACC TGT CAC AAA GGA GGC GAG	317
	Gly Asp Ala Gly Gln Tyr Thr Cys His Lys Gly Gly Glu	
	85 90 95	
45	GTT CTA AGC CAT TCG CTC CTG CTG CTT CAC AAA AAG GAA	356
	Val Leu Ser His Ser Leu Leu Leu Leu His Lys Lys Glu	
	100 105	
50	GAT GGA ATT TGG TCC ACT GAT ATT TTA AAG GAC CAG AAA	395
	Asp Gly Ile Trp Ser Thr Asp Ile Leu Lys Asp Gln Lys	
	110 115 120	

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	GAA	CCC	AAA	AAT	AAG	ACC	TTT	CTA	AGA	TGC	GAG	GCC	AAG	434
	Glu	Pro	Lys	Asn	Lys	<u>Thr</u>	<u>Phe</u>	<u>Leu</u>	<u>Arg</u>	Cys	Glu	Ala	Lys	
				125					130					
5	AAT	TAT	TCT	GGA	CGT	TTC	ACC	TGC	TGG	TGG	CTG	ACG	ACA	473
	Asn	Tyr	Ser	Gly	Arg	Phe	Thr	Cys	Trp	Trp	Leu	Thr	Thr	
	135					140					145			
	ATC	AGT	ACT	GAT	TTG	ACA	TTC	AGT	GTC	AAA	AGC	AGC	AGA	512
10	Ile	Ser	Thr	Asp	Leu	Thr	Phe	Ser	Val	Lys	Ser	Ser	Arg	
			150					155					160	
	GGC	TCT	TCT	GAC	CCC	CAA	GGG	GTG	ACG	TGC	GGA	GCT	GCT	551
	Gly	Ser	Ser	Asp	Pro	Gln	Gly	Val	Thr	Cys	Gly	Ala	Ala	
					165					170				
15	ACA	CTC	TCT	GCA	GAG	AGA	GTC	AGA	GGG	GAC	AAC	AAG	GAG	590
	Thr	Leu	Ser	Ala	Glu	Arg	Val	Arg	Gly	Asp	Asn	Lys	Glu	
		175					180					185		
	TAT	GAG	TAC	TCA	GTG	GAG	TGC	CAG	GAG	GAC	AGT	GCC	TGC	629
20	Tyr	Glu	Tyr	Ser	Val	Glu	Cys	Gln	Glu	Asp	Ser	Ala	Cys	
				190					195					
	CCA	GCT	GCT	GAG	GAG	AGT	CTG	CCC	ATT	GAG	GTC	ATG	GTG	668
	Pro	Ala	Ala	Glu	Glu	Ser	Leu	Pro	Ile	Glu	Val	Met	Val	
	200					205					210			
25	GAT	GCC	GTT	CAC	AAG	CTC	AAG	TAT	GAA	AAC	TAC	ACC	AGC	707
	Asp	Ala	Val	His	Lys	Leu	<u>Lys</u>	<u>Tyr</u>	<u>Glu</u>	<u>Asn</u>	<u>Tyr</u>	<u>Thr</u>	Ser	
			215					220					225	
	AGC	TTC	TTC	ATC	AGG	GAC	ATC	ATC	AAA	CCT	GAC	CCA	CCC	746
30	Ser	Phe	Phe	Ile	Arg	<u>Asp</u>	<u>Ile</u>	<u>Ile</u>	Lys	<u>Pro</u>	<u>Asp</u>	<u>Pro</u>	<u>Pro</u>	
					230					235				
	AAG	AAC	TTG	CAG	CTG	AAG	CCA	TTA	AAC	AAT	TCT	CGG	CAG	785
	<u>Lys</u>	Asn	Leu	Gln	Leu	Lys	Pro	Leu	Lys	Asn	Ser	Arg	Gln	
		240					245					250		
35	GTG	GAG	GTC	AGC	TGG	GAG	TAC	CCT	GAC	ACC	TGG	AGT	ACT	824
	Val	Glu	Val	Ser	Trp	Glu	Tyr	Pro	Asp	Thr	Trp	Ser	Thr	
				255					260					
	CCA	CAT	TCC	TAC	TTC	TCC	CTG	ACA	TTC	TGC	GTT	CAG	GTC	863
40	Pro	His	Ser	Tyr	Phe	Ser	Leu	Thr	Phe	Cys	Val	Gln	Val	
	265					270					275			
	CAG	GGC	AAG	AGC	AAG	AGA	GAA	AAG	AAA	GAT	AGA	GTC	TTC	902
	Gln	Gly	Lys	Ser	Lys	Arg	Glu	Lys	Lys	Asp	Arg	Val	Phe	
			280					285					290	

19

	ACG GAC AAG ACC TCA GCC ACG GTC ATC TGC CGC AAA AAT	941
	Thr Asp Lys Thr Ser Ala Thr Val Ile Cys Arg Lys <u>Asn</u>	
	295 300	
5	GCC AGC ATT AGC GTG CGG GCC CAG GAC CGC TAC TAT AGC	980
	<u>Ala Ser Ile Ser Val</u> Arg Ala Gln Asp Arg Tyr Tyr Ser	
	305 310 315	
10	TCA TCT TGG AGC GAA TGG GCA TCT GTG CCC TGC AGT TAG	1019
	Ser Ser Trp Ser Glu Trp Ala Ser Val Pro Cys Ser *	
	320 325	
	GTTCTGATCC AGGATGAAAA TTTGGAGGAA AAGTGGAAGA	1059
	TATTAAGCAA AATGTTTAAA GACACAACGG AATAGACCCA	1099
15	AAAAGATAAT TTCTATCTGA TTTGCTTTTAA AACGTTTTTTT	1139
	TAGGATCACA ATGATATCTT TGCTGTATTT GTATAGTTCTG	1179
	ATGCTAAATG CTCATTGAAA CAATCAGCTA ATTTATGTAT	1219
	AGATTTTCCA GCTCTCAAGT TGCCATGGGC CTTCATGCTA	1259
	TTTAAATATT TAAGTAATTT ATGTATTTAT TAGTATATTA	1299
20	CTGTTATTTA ACGTTTGTCT GCCAGGATGT ATGGAATGTT	1339
	TCATACTCTT ATGACCTGAT CCATCAGGAT CAGTCCCTAT	1379
	TATGCAAAAT GTGAATTTAA TTTTATTTGT ACTGACAACT	1419
	TTTCAAGCAA GGCTGCAAGT ACATCAGTTT TATGACAATC	1459
	AGGAAGAATG CAGTGTTCTG ATACCAGTGC CATCATACAC	1499
25	TTGTGATGGA TGGGAACGCA AGAGATACTT ACATGGAAAC	1539
	CTGACAATGC AAACCTGTTG AGAAGATCCA GGAGAACAAG	1579
	ATGCTAGTTC CCATGTCTGT GAAGACTTCC TGGAGATGGT	1619
	GTTGATAAAG CAATTTAGGG CCACTTACAC TTCTAAGCAA	1659
	GTTTAATCTT TGGATGCCTG AATTTTAAAA GGGCTAGAAA	1699
30	AAAATGATTG ACCAGCCTGG GAAACATAAC AAGACCCCGT	1739
	CTCTACAAAA AAAATTTAAA ATTAGCCAGG CGTGGTGGCT	1779

20

	CATGCTTGTG	GTCCCAGCTG	TTCAGGAGGA	TGAGGCAGGA	1819
	GGATCTCTTG	AGCCCAGGAG	GTCAAGGCTA	TGGTGAGCCG	1859
	TGATTGTGCC	ACTGCATACC	AGCCTAGGTG	ACAGAATGAG	1899
	ACCCTGTCTC	AAAAAAAAAA	ATGATTGAAA	TTAAAATTCA	1939
5	GCTTTAGCTT	CCATGGCAGT	CCTCACCCCC	ACCTCTCTAA	1979
	AAGACACAGG	AGGATGACAC	AGAAACACCG	TAAGTGTCTG	2019
	GAAGGCAAAA	AGATCTTAAG	ATTCAAGAGA	GAGGACAAGT	2059
	AGTTATGGCT	AAGGACATGA	AATTGTCAGA	ATGGCAGGTG	2099
	GCTTCTTAAC	AGCCATGTGA	GAAGCAGACA	GATGCAAAGA	2139
10	AAATCTGGAA	TCCCTTTCTC	ATTAGCATGA	ATGAACCTGA	2179
	TACACAATTA	TGACCAGAAA	ATATGGCTCC	ATGAAGGTGC	2219
	TACTTTTAAG	TAATGTATGT	GCGCTCTGTA	AAGTGATTAC	2259
	ATTTGTTTCC	TGTTTGTTTA	TTTATTTATT	TATTTTGTCA	2299
	TTCTGAGGCT	GAAC TAATAA	AAACTCTTCT	TTGTAATCAA	2339
15	AAAAAAAAAA	AAAAAACTCT	AGA		2362

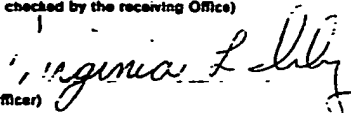
This cloned sequence in plasmid pNK40-4 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland on July 31, 1990 under accession number 40854. A prior partial clone
5 of this larger fragment, containing the N-terminal non-coding region through sequence containing up to approximately nucleotide #888, was called pNK-6 and deposited with the ATCC on February 3, 1989 under ATCC No. 40545. Another partial clone of the larger subunit,
10 pNK162 was sequenced and contains the sequence from nucleotides #643 to 2362 of Table I. This clone is maintained at the labs of Genetics Institute, Inc.

Two independent cDNA clones were identified which encode the sequence of the small (30-35 kD) subunit
15 of NKSF. The longer clone (designated p35nksf14-1-1) is shown in Table II. The shorter clone (designated p35nksf9-1-1) begins at nucleotide #133 (indicated by *) and ends at nucleotide #1335 (indicated by *) of Table II and the deposited sequence. Between those two
20 nucleotides, the smaller clone is identical to the sequence of Table II except for 5 nucleotide changes in the 3' non-coding region. This shorter clone thus has a coding sequence beginning with Met (amino acid #35) in Table II. The additional sequence at the 5' end of
25 p35nksf14-1-1 encodes an in-frame initiation codon (ATG) 34 residues 5' of the operative initiation codon in p35nksf9-1-1.

ANNEX M3

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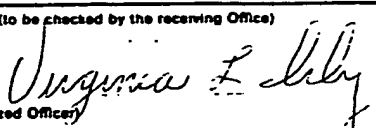
International Application No: PCT/

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>21</u> , line <u>4</u> of the description ¹	
A. IDENTIFICATION OF DEPOSIT ¹	
Further deposits are identified on an additional sheet <input type="checkbox"/> Plasmid DNA, NKSF 40 Kd Chain, pNK40-4	
Name of depositary institution ² American Type Culture Collection	
Address of depositary institution (including postal code and country) ² 12301 Parklawn Drive, Rockville, MD 20852 USA	
Date of deposit ² July 31, 1990	Accession Number ² 40854
B. ADDITIONAL INDICATIONS ³ (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ³ (if the indications are not for all designated States)	
All	
D. SEPARATE FURNISHING OF INDICATIONS ³ (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later ³ (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
 (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau ^{1*}	
was	
(Authorized Officer)	

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International Application No: PCT/

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MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>21</u> , line <u>9</u> of the description ¹	
A. IDENTIFICATION OF DEPOSIT ²	
Further deposits are identified on an additional sheet <input type="checkbox"/> Plasmid, pNK-6	
Name of depositary institution ⁴	
American Type Culture Collection	
Address of depositary institution (including postal code and country) ⁴	
12301 Parklawn Drive, Rockville, MD 20852 USA	
Date of deposit ⁵	Accession Number ⁶
February 3, 1989	40545
B. ADDITIONAL INDICATIONS ⁷ (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ⁸ (If the indications are not for all designated States)	
All	
D. SEPARATE FURNISHING OF INDICATIONS ⁹ (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later ⁹ (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
<div style="text-align: right;"> (Authorized Officer)</div>	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau ¹⁰	
was	
(Authorized Officer)	

Both of these clones ncode all of the peptide sequences identified in the tryptic digest of purified NKSF, which were not found in the 40 kD subunit protein, as well as the amino terminal sequence of the purified 30 kD subunit. These sequences are underlined in Table II. The clones contain the coding sequence for two possible versions of the 30-35 kD subunit of NKSF depending on whether translation begins with Met #1 or Met #35 in Table II. However, because the 30-35 kD protein subunit of NKSF is believed to be generated by cleavage following Ala (amino acid #56), both sequences should yield the same mature protein. The sequence of p35nksf14-1-1 was deposited with the ATCC on September 11, 1990 under accession number 40886.

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International Application No: PCT/

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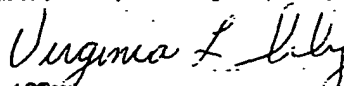
MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>22</u> , line <u>14</u> of the description ¹	
A. IDENTIFICATION OF DEPOSIT ²	
Further deposits are identified on an additional sheet <input type="checkbox"/> ³ Plasmid, p35 NKSF 14-1-1	
Name of depositary institution ⁴ American Type Culture Collection	
Address of depositary institution (including postal code and country) ⁵ 12301 Parklawn Drive, Rockville, MD 20852 USA	
Date of deposit ⁶ July 1, 1987	Accession Number ⁷ 40886
B. ADDITIONAL INDICATIONS ⁸ (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ⁹ (if the indications are not for all designated States)	
All	
D. SEPARATE FURNISHING OF INDICATIONS ¹⁰ (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later ¹¹ (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
<div style="text-align: right;"> (Authorized Officer)</div>	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau ¹²	
was <div style="text-align: right;">(Authorized Officer)</div>	

TABLE II

**Nucleotide and amino acid sequence of 30kD subunit
p35nksf14-1-1, long clone and p35nksf 9-1-1, short clone**

5	<u>GTCGACTCTA GAG</u> GTCACCGAGA AGCTGATGTA GAGAGAGACA	30
	polylinker * nucleotide #1 of p35nksf14-1-1	
	CAGAAGGAGA CAGAAAGCAA GAGACCAGAG TCCCGGGAAA	70
	GTCCTGCCGC GCCTCGGGAC AATTATAAAA	100
10	ATG TGG CCC CCT GGG TCA GCC TCC Met Trp Pro Pro Gly Ser Ala Ser	124
	1 5	
	* (nucleotide #1 of p35nksf9-1-1)	
15	CAG CCA CCG CCC TCA CCT GCC GCG GCC ACA GGT CTG CAT	163
	Gln Pro Pro Pro Ser Pro Ala Ala Ala Thr Gly Leu His	
	10 15 20	
	Pst I	
20	CCA GCG GCT CGC CCT GTG TCC <u>CTG CAG</u> TGC CGG CTC AGC	202
	Pro Ala Ala Arg Pro Val Ser Leu Gln Cys Arg Leu Ser	
	25 30	
	ATG TGT CCA GCG CGC AGC CTC CTC CTT GTG GCT ACC CTG	241
	Met Cys Pro Ala Arg Ser Leu Leu Leu Val Ala Thr Leu	
	35 40 45	
25	GTC CTC CTG GAC CAC CTC AGT TTG GCC AGA AAC CTC CCC	280
	Val Leu Leu Asp His Leu Ser Leu Ala <u>Arg Asn Leu Pro</u>	
	50 55 60	
30	GTG GCC ACT CCA GAC CCA GGA ATG TTC CCA TGC CTT CAC	319
	<u>Val Ala Thr Pro Asp Pro Gly Met Phe Pro</u> Cys Leu His	
	65 70	
35	CAC TCC CAA AAC CTG CTG AGG GCC GTC AGC AAC ATG CTC	358
	His Ser Gln Asn Leu Leu Arg <u>Ala Val Ser Asn Met Leu</u>	
	75 80 85	

24

	CAG	AAG	GCC	AGA	CAA	ACT	CTA	GAA	TTT	TAC	CCT	TGC	ACT	397
	<u>Gln</u>	<u>Lys</u>	Ala	Arg	Gln	Thr	Leu	Glu	Phe	Tyr	Pro	Cys	Thr	
				90					95					
5	TCT	GAA	GAG	ATT	GAT	CAT	GAA	GAT	ATC	ACA	AAA	GAT	AAA	436
	Ser	Glu	Glu	Ile	Asp	His	Glu	Asp	Ile	Thr	Lys	Asp	Lys	
	100				105						110			
10	ACC	AGC	ACA	GTG	GAG	GCC	TGT	TTA	CCA	TTG	GAA	TTA	ACC	475
	Thr	Ser	Thr	Val	Glu	Ala	Cys	Leu	Pro	Leu	Glu	Leu	Thr	
			115					120					125	
	AAG	AAT	GAG	AGT	TGC	CTA	AAT	TCC	AGA	GAG	ACC	TCT	TTC	514
	Lys	Asn	Glu	Ser	Cys	Leu	Asn	Ser	Arg	Glu	Thr	Ser	Phe	
					130					135				
15	ATA	ACT	AAT	GGG	AGT	TGC	CTG	GCC	TCC	AGA	AAG	ACC	TCT	553
	Ile	Thr	Asn	Glu	Ser	Cys	Leu	Ala	Ser	Arg	Lys	Thr	Ser	
		140					145					150		
	TTT	ATG	ATG	GCC	CTG	TGC	CTT	AGT	AGT	ATT	TAT	GAA	GAC	592
	Phe	Met	Met	Ala	Leu	Cys	Leu	Ser	Ser	Ile	Tyr	Glu	Asp	
				155					160					
20	TTG	AAG	ATG	TAC	CAG	GTG	GAG	TTC	AAG	ACC	ATG	AAT	GCA	631
	Leu	Lys	Met	Tyr	Gln	Val	Glu	Phe	Lys	Thr	Met	Asn	Ala	
	165					170					175			
25	AAG	CTT	CTG	ATG	GAT	CCT	AAG	AGG	CAG	ATC	TTT	CTA	GAT	670
	Lys	Leu	<u>Leu</u>	<u>Met</u>	<u>Asp</u>	<u>Pro</u>	<u>Lys</u>	Arg	Gln	Ile	Phe	Leu	Asp	
			180				185						190	
	CAA	AAC	ATG	CTG	GCA	GTT	ATT	GAT	GAG	CTG	ATG	CAG	GCC	709
	Gln	Asn	Met	Leu	Ala	Val	Ile	Asp	Glu	Leu	Met	Gln	Ala	
					195					200				
30	CTG	AAT	TTC	AAC	AGT	GAG	ACT	GTG	CCA	CAA	AAA	TCC	TCC	748
	Leu	Asn	Phe	Asn	Ser	Glu	Thr	Val	Pro	Gln	Lys	Ser	Ser	
		205					210					215		
35	CTT	GAA	GAA	CCG	GAT	TTT	TAT	AAA	ACT	AAA	ATC	AAG	CTC	787
	Leu	Glu	Glu	Pro	Asp	Phe	Tyr	Lys	Thr	Lys	Ile	Lys	Leu	
				220					225					
	TGC	ATA	CTT	CTT	CAT	GCT	TTC	AGA	ATT	CGG	GCA	GTG	ACT	826
	Cys	Ile	Leu	Leu	His	Ala	Phe	Arg	Ile	Arg	Ala	Val	Tyr	
	230					235					240			
40	ATT	GAT	AGA	GTG	ATG	AGC	TAT	CTG	AAT	GCT	TCC			860
	Ile	Asp	Arg	<u>Val</u>	<u>Met</u>	<u>Ser</u>	<u>Tyr</u>	<u>Leu</u>	<u>Asn</u>	<u>Ala</u>	Ser			
			245					250						

25

	TAAAAAAGCG AGGTCCCTCC AAACCGTTGT CATTTTATA	900
	AAACTTTGAA ATGAGGAAAC TTTGATAGGA TGTGGATTAA	940
	GAAC TAGGGA GGGGGAAAGA AGGATGGGAC TATTACATCC	980
	ACATGATACC TCTGATCAAG TATTTTGTAC ATTTACTGTG	1020
5	GATAAATTGT TTTTAAGTTT TCATGAATGA ATTGCTAAGA	1060
	AGGGAAAATA TCCATCCTGA AGGTGTTTTT CATTCACTTT	1100
	AATAGAAGGG CAAATATTTA TAAGCTATTT CTGTACCAAA	1140
	GTGTTTGTGG AAACAAACAT GTAAGCATAA CTTATTTTAA	1180
	AATATTTATT TATATAACTT GGTAATCATG AAAGCATCTG	1220
10	AGCTAACTTA TATTTATTTA TGTTATATTT ATTAAATTAT	1260
	TCATCAAGTG TATTTGAAAA ATATTTTAA GTGTTCTAAA	1300
	(last nucleotide of small clone, p35nksf)	
		*
	AATAAAAGTA TTGAATTAAA AAAAAAAAAA AAAAAAAAAA	1340
15		
		* polylinker
	AAAAAAAAAA AAAAAAAAAA AAAA <u>CCTG CAGCCCGGGG GATCC</u>	1364
		last nucleotide in clone sequence

Sequence from p35nksf9-1-1 (from the Pst I site underlined in Table II to the Pst I site in the Bluescript polylinker sequence), when introduced into Cos cells in the expression vector pEMC3(1) along with a
5 plasmid expressing the 40 kD subunit, yielded biologically active NKSF. This material was active in the same bioassays used to test natural NKSF as discussed below. This sequence may be obtained from p35nksf14-1-1 by digestion with Pst I. Alternatively the cloned
10 sequence of plasmid p35nksf9-1-1, containing the shorter 30-35 kD subunit sequence, is being maintained at the laboratories of Genetics Institute, CambridgePark, MA and will be made available to the public upon grant of the patent.

15 A cDNA suitable for expression of the longer version of the 30-35 kD subunit may be obtained from the p35nksf14-1-1 deposited clone by digestion with SalI and NotI. The longer 30-35 kD subunit contains an earlier Met (amino acid #1) codon, additional 5' coding and non-
20 coding sequences as well as 3' non-coding sequence. The sequence from Met (amino acid #35) to the N-terminus of the mature protein (encoded by both cDNAs) encodes a sequence which resembles a signal peptide and may direct the proper folding and/or secretion of the subunit. It
25 is therefore possible that the longer 30-35 kD subunit sequence may be more efficiently expressed and secreted

by the Cos cells than the shorter version. It may also fold differently, thereby conferring NKSF activity independent of the presence of the 40 kD subunit.

Table II indicates the placement of polylinker sequence in the deposited clone, as well as the first and last nucleotide of the larger and smaller versions of this subunit. Also indicated in the sequence are the 5' PstI site for obtaining the sequence of the small subunit which has been expressed and underlined tryptic fragment sequences.

Allelic variations of DNA sequences encoding the peptide sequences and the large and small subunits described above are also included in the present invention as well as analogs or derivatives thereof.

Thus the present invention also encompasses these novel DNA sequences, free of association with DNA sequences encoding other primate proteins, and coding on expression for NKSF polypeptides, including those of its large and small subunits. These DNA sequences include those containing one or more of the above-identified DNA and peptide sequences and those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequences. An example of one such stringent hybridization condition is hybridization at 4XSSC at

65°C, followed by a washing in 0.1XSSC at 65°C for an hour. Alternatively an exemplary stringent hybridization condition is in 50% formamide, 4XSSC at 42°C.

5 DNA sequences which hybridize to the sequences for NKSF or its subunits under relaxed hybridization conditions and which code on expression for NKSF peptides having NKSF biological properties also encode novel NKSF polypeptides. Examples of such non-stringent
10 hybridization conditions are 4XSSC at 50°C or hybridization with 30-40% formamide at 42°C. For example, a DNA sequence which shares regions of significant homology, e.g., sites of glycosylation or disulfide linkages, with the sequences of NKSF and
15 encodes a protein having one or more NKSF biological properties clearly encodes a NKSF polypeptide even if such a DNA sequence would not stringently hybridize to the NKSF sequences.

Similarly, DNA sequences which code for NKSF
20 polypeptides coded for by the sequence of NKSF, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) are also encompassed
25 by this invention. Variations in the DNA sequence of NKSF which are caused by point mutations or by induced

modifications to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention.

NKSF polypeptides may also be produced by known conventional chemical synthesis. Methods for constructing the polypeptides of the present invention by synthetic means are known to those of skill in the art. The synthetically-constructed NKSF polypeptide sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with NKSF polypeptides may possess NKSF biological properties in common therewith. Thus, they may be employed as biologically active or immunological substitutes for natural, purified NKSF polypeptides in therapeutic and immunological processes.

The NKSF polypeptides provided herein also include factors encoded by sequences similar to those of purified homogeneous and recombinant NKSF protein, or the subunit polypeptides, but into which modifications are naturally provided or deliberately engineered. Modifications in the peptides or DNA sequences can be made by one skilled in the art using known techniques. Modifications of interest in the NKSF sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequences.

Mutagenic techniques for such replacement, insertion or deletion are well known to one skilled in the art. [See, e.g., United States patent 4,518,584.]

Other specific mutations of the sequences of
5 the NKSF polypeptide or the subunit polypeptides described herein may involve modifications of a glycosylation site. The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked
10 glycosylation recognition site or at any site of the molecule that is modified by addition of O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular
15 glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation
20 recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence.

Expression of such altered nucleotide sequences produces variants which are not glycosylated at that
25 site.

Other analogs and derivatives of the sequence of NKSF or of its subunits which would be expected to retain NKSF activity in whole or in part may also be easily made by one of skill in the art given the disclosures herein. One such modification may be the attachment of polyethylene glycol onto existing lysine residues or the insertion of a lysine residue into the sequence by conventional techniques to enable the attachment. Such modifications are believed to be encompassed by this invention.

The present invention also provides a method for producing NKSF polypeptides. The method of the present invention involves culturing a suitable cell or cell line, which has been transformed with a DNA sequence coding on expression for an NKSF polypeptide or subunit, under the control of known regulatory sequences. Preferably DNA sequences for both subunits are transformed into a host cell.

Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et

al, U. S. Patent 4,419,446. Expression of two different DNAs simultaneously in CHO cells has been described, for example, in published PCT International Application WO88/08035. Other suitable mammalian cell lines, are the
5 monkey COS-1 cell line, and the CV-1 cell line, originally developed at the Wistar Institute, Philadelphia, Pennsylvania.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the
10 various strains of E. coli (e.g., HB101, MC1061 and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

15 Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention.
20 See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

The present invention also provides vectors for use in the method of expression of novel NKSF polypeptides. These vectors contain the novel NKSF DNA
25 sequences which code for NKSF polypeptides of the invention, including the subunit polypeptides.

Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention and useful in the production of NKSF polypeptides. The vector employed in the method also
5 contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and capable of directing the replication and expression thereof in selected host cells.

Thus NKSF, purified to homogeneity from cell
10 sources or produced recombinantly or synthetically, may be used in a pharmaceutical preparation or formulation to treat cancer or other disease states which respond to enhanced NK cell activity or increased in vivo production of gamma interferon or GM-CSF. Such pathological states
15 may result from disease, exposure to radiation or drugs, and include for example, leukopenia, bacterial and viral infections, anemia, B cell or T cell deficiencies including immune cell or hematopoietic cell deficiency following a bone marrow transplantation. Therapeutic
20 treatment of cancer and other diseases with these NKSF polypeptide compositions may avoid undesirable side effects caused by treatment with presently available drugs. The NKSF polypeptide compositions according to the present invention may also be used in the treatment of
25 Acquired Immunodeficiency Syndrome (AIDS) and other viral infections, particularly non-responsive viral infections, as well as bacterial infections.

It may also be possible to employ one or both of the subunit polypeptides of NKSF or peptide fragments thereof in such pharmaceutical formulations.

5 The polypeptides of the present invention may also be employed, alone or in combination with other cytokines, hematopoietins, interleukins, growth factors or antibodies in the treatment of cancer or other disease states. For example, NKSF polypeptides have been shown to have a synergistic effect when administered in
10 connection with IL-2. This is expected to be useful in the treatment of infections, particularly viral infections and cancers. Other uses for these novel polypeptides are in the development of monoclonal and polyclonal antibodies generated by standard methods for
15 diagnostic or therapeutic use.

Therefore, as yet another aspect of the invention are methods and therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount
20 of the NKSF protein or a subunit polypeptide or therapeutically effective fragment thereof of the present invention in admixture with a pharmaceutically acceptable carrier. This composition can be systemically administered parenterally. Alternatively, the
25 composition may be administered intravenously. If desirable, the composition may be administered

subcutaneously. When systematically administered, the therapeutic composition for use in this invention is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a
5 pharmaceutically acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

The dosage regimen involved in a method for treating the above-described conditions will be
10 determined by the attending physician considering various factors which modify the action of drugs, e.g. the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen
15 should be in the range of 1-1000 micrograms of NKSF protein or subunit thereof or 50 to 5000 units (i.e., one unit per ml being the concentration of protein which leads to half maximal stimulation in the gamma interferon induction assay) of protein per kilogram of body weight.

20 The therapeutic method and compositions of the present invention may also include co-administration with other human factors. Exemplary cytokines or hematopoietins for such use include the known factors IL-1, IL-2 and IL-6 particularly. [See, e.g., PCT
25 publications WO85/05124, and WO88/00206; and European

patent application 0,188,864.]. Other potential candidates for participation in NKSF therapy may also include IL-4, G-CSF, CSF-1, GM-CSF, IL-3, IL-11 or erythropoietin. Growth factors like B cell growth factor, B cell differentiation factor, or eosinophil differentiation factors may also prove useful in co-administration with NKSF.

Similarly, administration of NKSF or a subunit or fragment thereof with or prior to administration of an antibody capable of binding to the Fc receptor on NK cells may enhance ADCC therapy directed against tumors. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

The following examples illustratively describe the purification and characteristics of homogeneous human NKSF and other methods and products of the present invention. These examples are for illustration and do not limit the scope of the present invention.

Example 1. Preparation of Serum-Free RPMI 8866 Cell-
Conditioned Medium

The human B-lymphoblastoid cell line RPMI 8866 was maintained in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS). For preparation of serum free conditioned medium, cells were washed and suspended (10^6 cells/ml) in serum free RPMI 1640 medium containing 10^{-7} M phorbol-12-13-dibutyrate (PdbU) and cultured for 48 hours at 37°C, 5% CO₂. The cell free supernatants were harvested by filtration through a 0.2 μ m filter [Durapore® hydrophilic cartridge filter, Millipore, Bedford, MA], and Tween-20 and phenylmethylsulfonyl-fluoride (PMSF) were added to 0.02% and 0.1 mM, respectively. The cell conditioned medium was then concentrated 50 fold under pressure using an ultra-filtration cartridge [Spiral-Wound, S1, Amicon, Danvers, MA].

Example 2. Purification of NKSF from Conditioned Medium

The following procedures are presently employed to obtain homogeneous NKSF protein from RPMI 8866 conditioned medium, as described in Example 1 above.

a. Anion Exchange Cartridge Chromatography

Two liters of the crude concentrated conditioned medium was diluted with distilled water to a conductivity of 6m Os/cm and adjusted to pH 8 with 1 M Tris-HCl buffer (pH 8). The concentrate was then applied to five QAE Zetaprep 250 cartridges [Pharmacia] connected in parallel and previously equilibrated with 0.1 M Tris-HCl buffer (pH 8) at a flow rate of 150 ml/min. Unless otherwise cited, all the buffers used for purification contained 0.02% Tween-20 and 0.1 mM PMSF. The cartridges were washed with 3 liters of 0.1 M Tris-HCl buffer (pH 6.8) followed by washing with 1.5 liters of 0.5 M NaCl in 0.1 M Tris-HCl buffer (pH 6.8) and 300 ml fractions were collected. The NKSF activity was eluted with the 0.5 M NaCl-containing wash.

b. Lentil-Lectin Sepharose Chromatography

Pooled NKSF-containing fractions from two separate QAE Zetaprep elutions were pooled and applied directly to a column (2.5x15 cm) of lentil-lectin Sepharose 4B [Pharmacia] which has been equilibrated with 20 mM Tris-HCl buffer (pH 7.2). After washing with five column volumes of equilibration buffer, the column was eluted with three column volumes of 20 mM Tris-HCl buffer (pH 7.2) containing 0.2 M α -methyl-D-mannopyranoside [Sigma] and 0.5 M NaCl. Approximately half of the NKSF activity was bound by the column and was recovered in the fractions eluted with α -methyl-D-mannopyranoside.

c. Hydroxylapatite Chromatography

Concentrated material from the pool of NKSF activity which bound to the lentil-lectin Sepharose column was dialyzed against 1 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM CaCl_2 and 0.15 M NaCl and applied to a Biogel HT [BioRad] column (2x5 cm) previously equilibrated with 1 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM CaCl_2 . The column was washed with five column volumes of equilibration buffer and eluted with 100 ml of a linear gradient from 1 mM to 400 mM potassium phosphate buffer (pH 6.8) containing 0.15 M NaCl. 4 ml fractions were collected and tested for NKSF activity. A single peak of activity emerged from the column between the approximately 200 mM and 300 mM potassium phosphate.

d. Heparin Sepharose Chromatography

Eluted NKSF-containing fractions from the Biogel HT column were pooled and dialyzed against 20 mM sodium phosphate buffer (pH 7.2) and applied to a Heparin Sepharose [Pierce, Rockford, IL] column (1x10 cm). The column was washed with five column volumes of 20 mM sodium phosphate buffer (pH 7.2) and eluted with the same buffer containing 1 M NaCl. 3 ml fractions were collected and NKSF activity measured. Essentially all of the activity was bound by the Heparin column and recovered in the 1 M NaCl wash.

e. Mono Q Chromatography

Pooled fractions from the Heparin

Sepharose column were dialyzed against 20 mM Tris-HCl
buffer (pH 6.8) containing 1% ethylene glycol and 0.1 mM
5 PMSF but no Tween-20 (buffer A) and concentrated to 2 ml
using a stirred cell [Amicon] with a YM 10 membrane. The
sample was applied to a Mono Q (5/5) column [Pharmacia-
FPLC apparatus] and eluted with a linear gradient from 0
M to 1 M NaCl in buffer A (pH 6.8). 0.5 ml fractions
10 were collected and tested for NKSF activity. The
activity emerged from the column as a single peak between
approximately 220 mM and 270 mM NaCl.

f. Gel Filtration Chromatography

Pooled fractions containing NKSF activity
15 from the Mono Q column were concentrated to 100
microliters by Speedvac Concentrator [Savant,
Farmingdale, NY] and applied to a FPLC Superose 12
column. Chromatography was run with 50 mM sodium
phosphate buffer (pH 7.2) containing 0.15 M NaCl, 1%
20 ethylene glycol and 0.1 mM PMSF. Flow rate was 0.6
ml/minute and 0.5 ml fractions were collected. NKSF
protein (70 kD) was separated from the approximately 37
kD protein contaminant.

Alternatively, the pooled Mono-Q fractions may be subjected to reverse-phase HPLC (C8 column) prior to the step (f) described above, to separate the protein contaminant from the active 70 kD protein.

5 Example 3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli [Laemmli, U. K., Nature, 227:680-685 (1970)] on 10% acrylamide slab gels (0.75 mm thickness). After
10 electrophoresis the gels were either stained by the silver-nitrate method using a silver staining reagents [BioRad] or cut into 2 mm slices and eluted in 0.5 ml RPMI medium for 4 hours at 24°C and assayed for NKSF activity. Apparent molecular weight was determined with
15 protein standards, phospholipase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD) and lactalbumin (14.4 kD).

SDS-PAGE analysis (non-reducing conditions) of
20 the Mono Q column fractions (Example 2, step (e)) beginning with several fractions which eluted before the NKSF activity, continuing right through the active fractions and ending with fractions which eluted after the peak of NKSF activity, revealed that the presence of
25 two proteins (70 kD and 37 kD) correlated with the

presence of NKSF activity in the various Mono Q fractions. The active fractions were rerun on a second non-reducing gel and the proteins were eluted from the regions corresponding to the 70 kD and 37 kD bands and tested for NKSF activity. The activity all correlated with the 70 kD species indicating that this protein is NKSF.

The 70 kD species was eluted from the gel, iodinated using chloramine T [Sigma, St. Louis, MO] and rerun on a second SDS gel after boiling for two minutes in the presence of the reducing agent, β -mercaptoethanol (10%). Under these conditions, the 70 kD species resolved into two distinct subunits of molecular weights 40kD and 30kD, indicating that the native NKSF may be a disulfide-bonded heterodimer of these subunit polypeptides. Alternatively, NKSF may be a dimer formed by multiples of the larger or smaller subunits. The reduction of the native 70kD NKSF appeared to destroy all of its ability to induce peripheral blood lymphocyte production of gamma interferon.

Example 4. Recovery of Protein

Starting with 500 liters of RPMI 8866 cell-free conditioned medium, the final pooled active fractions from the Mono Q column contained approximately 10 μ g of protein, estimated from the intensities of silver

staining by control proteins analyzed in parallel on the same gel. Approximately 6 μ g of this corresponded to the 70 kD NKSF protein. The estimated specific activity of the 70 kD NKSF is 1×10^7 u/mg. The overall recovery of NKSF activity in the preparation was 2%.

Example 5. NKSF Protein Composition

Homogeneous NKSF is reduced as described in the SDS-PAGE example above and digested with trypsin. Alternatively, non-reduced NKSF may be obtained from a reverse-phase HPLC column and digested with trypsin. Nine tryptic fragments are isolated having the following amino acid sequences:

- Fragment 1 - Leu-Thr-Ile-Gln-Val
- Fragment 2 - Lys-Tyr-Glu-Asn-Tyr-Thr
- Fragment 3 - Ile-Trp-Glu-Leu-Lys
- Fragment 4 - Leu-Met-Asp-Pro-Lys
- Fragment 5 - Val-Met-Ser-Tyr-Leu-Asn-Ala
- Fragment 6 - Ala-Val-Ser-Asn-Met-Leu-Gln-Lys
- Fragment 7 - Asn-Ala-Ser-Ile-Ser-Val
- Fragment 8 - Thr-Phe-Leu-Arg
- Fragment 9 - Asp-Ile-Ile-Lys-Pro-Asp-Pro-Pro-Lys.

Additionally, the amino acid sequences of the amino termini of each subunit of NKSF were determined from the isolated 40 kD and 30 kD species of NKSF after reduction, as described in Example 3. The amino terminal

5 sequence from the 40kD subunit was as follows:

Ile-Trp-Glu-Leu-Lys-Lys-Asp-Val-Tyr-Val-Val-Glu-Leu-Asp-Trp-Tyr-Pro-Asp-Ala-Pro-Gly-Glu-Met. The amino terminal sequence above as well as Fragments 1-3 and 7-9 proved to

10 be derived from the amino acid sequence of the clone of larger subunit identified in Table I above.

The amino terminal sequence from the 30kD smaller subunit was as follows: Arg-Asn-Leu-Pro-Val-Ala-Thr-Pro-Asp-Pro-Gly-Met-Phe-Pro. Fragments 4, 5 and 6 proved to be derived from the amino acid sequence of the

15 clone of the smaller subunit identified in Table II above.

Probes consisting of pools of oligonucleotides or unique oligonucleotides are designed according to the method of R. Lathe, J. Mol. Biol., 183(1):1-12 (1985).

20 The oligonucleotide probes are synthesized on an automated DNA synthesizer.

Because the genetic code is degenerate (more than one codon can code for the same amino acid) a mixture of oligonucleotides must be synthesized that

25 contains all possible nucleotide sequences encoding the

amino acid sequence of the tryptic fragment. It may be possible in some cases to reduce the number of oligonucleotides in the probe mixture based on codon usage because some codons are rarely used in eukaryotic genes, and because of the relative infrequency of the dinucleotide CpG in eukaryotic coding sequences [see J. J. Toole et al, Nature, 312:342-347 (1984)]. The regions of the amino acid sequences used for probe design are chosen by avoiding highly degenerate codons where possible. The oligonucleotides are synthesized on an automated DNA synthesizer and the probes are then radioactively labelled with polynucleotide kinase and ³²P-ATP.

A cDNA encoding the small subunit of NKSF was identified by screening a cDNA library (prepared in lambda Zap; Stratagene cloning systems, La Jolla, CA) made from polyadenylated RNA from PdBu induced 8866 cells (Univ. of Pennsylvania Cell Center) using established techniques (see Toole et al cited above). The screening was carried out using oligonucleotides with sequence predicted by those tryptic peptides not contained within the previously cloned cDNA coding for the 40kD protein as probes. Recombinants from this library are plated and duplicate nitrocellulose replicas made of the plates. The oligonucleotides are kinased with ³²P gamma ATP and hybridized to the replicas.

In particular two pools of oligonucleotides were synthesized based on the peptide Val-Met-Ser-Tyr-Leu-Asn-Ala. The sequences in one pool of 17mers were derived from the peptide sequence Met-Ser-Tyr-Leu-Asn-Ala and those in the second from Val-Met-Ser-Tyr-Leu-Asn. Clones which hybridized to the first pool of oligonucleotides were hybridized with the second pool. Hybridizations were performed at 48°C in a buffer containing 3M TMAC. Filters were subsequently washed in 3M TMAC, 50mM Tris pH 8 at 50°C. [See K. A. Jacobs et al, Nucl. Acids Res., 16:4637-4650 (1988).] Duplicate positives were plaque purified. Two clones were identified which hybridized to both pools, p35nksf9-1-1, and p35nksf14-1-1, described above.

The sequence and computer translations of cDNA clone p35nksf14-1-1 is shown in Table II. It includes all the peptide sequences identified in tryptic digests of purified NKSF not found in the 40kD subunit protein (underlined) as well as the amino terminal sequence of the purified 30kD subunit (underlined).

To obtain a full length cDNA clone for the 40kD subunit of NKSF, cDNA that had been previously prepared from 8866 polyadenylated RNA was cloned into λZAP as described above. Two hundred thousand recombinants from this library were plated, duplicate nitrocellulose filters were prepared and screened with a random primed

³²P labeled DNA fragment, the sequence of which is within pNK-6. The probing was done using standard stringent hybridization and washing conditions. Three duplicate positive plaques resulted from this screen. The plaques
5 were replated and reprobed using the above probe and conditions to clonally isolate the plaques. The three isolates were then probed with a ³²P end-labeled oligo dT probe (pd(T)₁₂₋₁₈, Pharmacia). This hybridization was done in 6XSSC, 5x Denhardt's solution, and carrier DNA plus
10 labeled probe at room temperature. One of the three isolates, pNK162, hybridized to the oligo dT probe and was sequenced.

Using standard restriction digestion and subcloning techniques, NKSF clones pNK-6 and pNK162 were
15 subcloned together in frame for transcription and translation and ligated into the pXM expression vector for COS expression. The resultant clone, pNK40-4 (Table I) is believed to contain the full length cDNA for the 40 kD NKSF subunit.

20 Example 6. Expression of Recombinant Human NKSF

To produce NKSF, the DNAs encoding its subunits are transferred into appropriate expression vectors, of which numerous types are known in the art for mammalian, insect, yeast, fungal and bacterial expression, by

standard molecular biology techniques. One such vector for mammalian cells is pXM [Y. C. Yang et al, Cell, 47:3-10 (1986)]. This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and the adenovirus VA I gene, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells [See, e.g., Kaufman, Proc. Natl. Acad. Sci. USA, 82:689-693 (1985)]. The pXM vector is linearized with the endonuclease enzyme XhoI and subsequently ligated in equimolar amount separately to the cDNA encoding the NKSF subunits that were previously modified by addition of synthetic oligonucleotides [Collaborative Research, Lexington, MA] that generate Xho I complementary ends to generate constructs for expression of each subunit of NKSF.

Another vector for mammalian expression, pEMC3(1) can be made by simple modification of the pEMC2B1 vector, described below. pEMC3(1) differs from pEMC2B1 by three restriction sites, SmaI, SalI, XbaI, in the polylinker region. To make pEMC3(1), these three restriction sites are inserted between the PstI and EcoRI restriction sites of pEMC2B1 by conventional means.

pEMC2B1 may be derived from pMT2pc which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under Accession Number ATCC 40348. The DNA is linearized by digestion of the plasmid with PstI. The DNA is then blunted using T₄ DNA polymerase. An oligonucleotide 5' TGCAGGCGAGCCTGAA TTCCTCGA 3' is then ligated into the DNA, recreating the PstI site at the 5' end and adding an EcoRI site and XhoI site before the ATG of the DHFR cDNA. This plasmid is called pMT21. pMT21 is cut with EcoRI and XhoI which cleaves the plasmid at two adjacent cloning sites. An EMCV fragment of 508 base pairs was cut from pMT₂ECAT₁ [S. K. Jong et al, J. Virol., 63:1651-1660 (1989)] with the restriction enzymes EcoRI and TaqαI. A pair of oligonucleotides 68 nucleotides in length were synthesized to duplicate the EMCV sequence up to the ATG. The ATG was changed to an ATT, and a C is added, creating a XhoI site at the 3' end. A TaqαI site is situated at the 5' end. The sequences of the oligonucleotides were: 5' CGAGGTTAAAAACGTCTAGGCCCCCGAACCACGGGGACGTGGTTCCTTT GAAAAACACGATTGC 3' and its complementary strand.

Ligation of the pMT21 EcoRI-to-XhoI fragment to the EMCV EcoRI-to-TaqαI fragment and to the TaqαI/XhoI oligonucleotides produced the vector pEMC2B1. This vector contains the SV40 origin of replication and

enhancer, the adenovirus major late promoter, a cDNA copy of the majority of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and the adenovirus VA I gene, DHFR and β -lactamase markers and an EMC sequence, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells.

The two different cDNAs are expressed simultaneously in the same host or independently in different hosts. In the latter case, the subunits are purified separately and the final active NKSF is assembled by renaturation of the individual subunits.

a. Mammalian Cell Expression

To obtain expression of the NKSF protein for use in the assays described below, the constructs containing the cDNAs for the 40 kD and 30 kD (smaller version) subunits were cloned separately into the mammalian expression vector pEMC3(1) and together introduced into COS cells by calcium phosphate coprecipitation and transfection. ³⁵S methionine labelled proteins (4hr pulse, 2 days after transfection) of approximately 80kD (nonreduced) and 40kD and 30kD (reduced) are present in PAGE gels of COS cotransfectant conditioned medium but not in negative control transfectants. The conditioned media from the COS cotransfectants collected 48 hrs after transfection, was

active in the gamma interferon (IFN γ) induction assay (see Example 7a).

Further evidence that the activity was identical to that purified from 8866 conditioned medium comes from the observations that the cotransfected conditioned media synergizes with IL2 in the IFN γ induction assay and that polyclonal rabbit antiserum (1:100 dilution) to the NKSF heavy chain, blocks the activity in the cotransfectant as well as RPMI 8861 conditioned medium. The antiserum was produced by immunizing rabbits with NKSF heavy chain purified from conditioned media from COS cells transfected with the NKSF heavy chain cDNA (cloned in pEMC 3(1)).

When the pNK40-4 plasmid was separately transfected into COS cells, the supernatant was collected and assayed, and the cells pulse labeled with ^{35}S cysteine. The labeled protein was run on an 11% acrylamide gel under standard reducing and nonreducing conditions. The unlabeled supernatant from this transfection with pNK40-4 was inactive in the gamma interferon induction assay and in the cell cytotoxicity assay, which were performed as described below in Example 8.

The mammalian cell expression vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors, e.g. replicons, selection genes, enhancers, promoters, and the like, may be obtained from natural sources or synthesized by known procedures. See, Kaufman et al, J. Mol. Biol., 159:511-521 (1982); and Kaufman, Proc. Natl. Acad. Sci., USA, 82:689-693 (1985). Exemplary mammalian host cells include particularly primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Candidate cells need not be genotypically deficient in the selection gene so long as the selection gene is dominantly acting. For stable integration of the vector DNAs, and for subsequent amplification of the integrated vector DNAs, both by conventional methods, CHO cells may be employed. Alternatively, the vector DNA may include all or part of the bovine papilloma virus genome [Lusky et al, Cell, 36:391-401 (1984)] and be carried in cell lines such as C127 mouse cells as a stable episomal element. Other suitable mammalian cell lines include but are not limited to, HeLa, COS-1 monkey cells, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Where the two subunits require simultaneous expression in mammalian cells, the two cDNAs may be introduced into the cells using two different selectable genes or markers. As discussed below in Example 7, this can readily be achieved in CHO cells using the dihydrofolate reductase (DHFR) gene as one marker and adenosine deaminase (ADA) as the other marker. Any combination of two genes which can be independently selected in any mammalian cell line are useful for this purpose. For example, a CHO cell line is independently developed for expression of one subunit under ADA selection and a different cell line is developed for expression of the other subunit under DHFR selection. The cell lines are fused in polyethylene glycol under double selection to yield stable lines expressing both subunits. Alternatively, the DNAs are introduced simultaneously or sequentially into the same cells, thereby yielding lines expressing active NKSF.

It is also possible that multicistronic vectors encoding both subunits with a single selectable marker might yield cells in which both subunits can be coamplified with one selective drug. Additionally, this effect may be achieved by simple cotransfection of cells simultaneously with separate vectors.

Stable transformants are then screened for expression of the product by standard immunological, biological or enzymatic assays. The presence of the DNA and mRNA encoding the NKSF polypeptides may be detected
5 by standard procedures such as Southern blotting and RNA blotting. Transient expression of the DNA encoding the polypeptides during the several days after introduction of the expression vector DNA into suitable host cells, such as COS-1 monkey cells, is measured without selection
10 by activity or immunologic assay of the proteins in the culture medium.

One skilled in the art can also construct other mammalian expression vectors comparable to the pEMC3(1) vector by, e.g., inserting the DNA sequences of the NKSF
15 subunits from the respective plasmids with appropriate enzymes and employing well-known recombinant genetic engineering techniques and other known vectors, such as pXM, pJL3 and pJL4 [Gough et al., EMBO J., 4:645-653 (1985)] and PMT2 (starting with PMT2-VWF, ATCC #67122;
20 see PCT application PCT/US87/00033). The transformation into appropriate host cells of these vectors with both NKSF subunits (either as separate vecotrs or in the same vector) can result in expression of the NKSF polypeptides.

b. Bacterial Expression Systems

Similarly, one skilled in the art could manipulate the sequences encoding the NKSF subunits by eliminating any mammalian regulatory sequences flanking the coding sequences and inserting bacterial regulatory sequences to create bacterial vectors for intracellular or extracellular expression of the NKSF subunits of the invention by bacterial cells. The DNA encoding the NKSF polypeptides may be further modified to contain different codons to optimize bacterial expression as is known in the art. Preferably the sequences encoding the mature NKSF subunits are operatively linked in-frame to nucleotide sequences encoding a secretory leader polypeptide permitting bacterial expression, secretion and processing of the mature NKSF polypeptides, also by methods known in the art. The simultaneous expression of both subunits of NKSF in E. coli using such secretion systems is expected to result in the secretion of the active heterodimer. This approach has yielded active chimeric antibody fragments [See, e.g., Bitter et al, Science, 240:1041-1043 (1988)].

Alternatively, the individual subunits are expressed in the mature form separately from the two different cDNAs in E. coli using vectors for intracellular expression and the subunits are isolated

separately, mixed and refolded by procedures well known in the art. See, for example, U.S. Patent 4,512,922. The compounds expressed through either route in bacterial host cells may then be recovered, purified, and/or
5 characterized with respect to physicochemical, biochemical and/or clinical parameters, all by known methods.

c. Insect or Yeast Cell Expression

Similar manipulations can be performed for
10 the construction of an insect vector for expression of NKSF polypeptides in insect cells [See, e.g., procedures described in published European patent application 155,476]. If the NKSF subunits are derived from a single cDNA, this cDNA will be expressed in insect cells.
15 Alternatively, if the NKSF subunits are derived from two different cDNAs, each subunit is separately inserted into an insect cell vector and the two resulting vectors co-introduced into insect cells to express biologically active NKSF.

20 Similarly yeast vectors are constructed employing yeast regulatory sequences to express either the individual NKSF subunits simultaneously, or, if the protein is derived from a single precursor, the cDNA encoding that precursor, in yeast cells to yield secreted
25 extracellular active NKSF heterodimer. Alternatively the individual subunits may be expressed intracellularly in

yeast, the individual polypeptides isolated and finally, refolded together to yield active NKSF. [See, e.g., procedures described in published PCT application WO 86/00639 and European patent application EP 123,289.]

5 Example 7. Construction of CHO Cell Lines Expressing High Levels of NKSF

One method for producing high levels of the NKSF protein of the invention from mammalian cells involves the construction of cells containing multiple
10 copies of the two cDNAs encoding the individual NKSF subunits.

Because the two NKSF polypeptides are each derived from separate mRNAs, each corresponding cDNA must be expressed simultaneously in CHO cells. Two different
15 selectable markers, e.g., DHFR and ADA, may be employed. One of the cDNAs is expressed using the DHFR system [Kaufman and Sharp, J. Mol. Biol., (1982) supra.] using, e.g., the vector pEMC3(1) to express one of the NKSF subunits and DHFR. The second subunit is expressed using
20 a second vector, e.g. pMT3SV2ADA [R. J. Kaufman, Meth. Enzymol., 185:537-566 (1990)]. Plasmid pMT3SV2ADA also directs the expression of ADA in mammalian cells. The first vector construct containing one subunit is

transfected into DHFR-deficient CHO DUKX-BII cells. The second vector construct containing the second subunit is transfected into a second CHO cell line. The transfected cells are selected for growth in increasing concentrations of methotrexate beginning with approximately 5nM with subsequent step-wise increments up to 100 μ M for the DHFR marker, or in 2'-deoxycoformycin (dCF) for the ADA marker beginning with 100 nM with subsequent step-wise increments up to 10 μ M. The expression of the individual cDNAs (one subunit under DHFR selection in one cell line and the other subunit under ADA selection in a second cell line) is assayed through a combination of mRNA blotting to test for transcription and immunoanalysis to test for protein production. The cells which express one of the subunits under ADA selection and the cells which express the other subunit under DHFR selection are finally fused in polyethylene glycol using methods well established in the art to yield a single cell line, resistant to both dCF and MTX and expressing both subunits to yield biologically active NKSF.

Another presently preferred method of expression is based on the development of a single cell line expressing both subunits. A first vector containing

one subunit, e.g., the first vector described above, is transfected into a selected CHO cell line and the expression of the subunit is amplified under drug selection as described above. Thereafter the second

5 vector containing the other subunit is transfected into the cell line which already contains amplified first vector. The cDNA expressing the other subunit, e.g., the second vector described above, may be introduced under a second drug selection. The second vector is then

10 amplified by the same techniques, resulting in a single cell line expressing both subunits simultaneously. (See, e.g., published PCT International Application W088/08035 for an exemplary description of independently amplifying a first gene linked to a DHFR gene and a second gene

15 linked to an ADA gene.)

In another method, two vectors constructs may be designed, e.g., a pEMC3(1) construct containing one subunit and the DHFR gene and a second pEMC3(1) construct containing the second subunit and the DHFR gene. The two

20 pEMC3(1) constructs expressing both NKSF subunits may be mixed and the mixture transfected into CHO cells. The cells are then amplified in MTX as described above to obtain a cell line producing both subunits. Alternatively two drug markers may be employed in this

method and the combined selection of both drugs may be used and transformants tested for NKSF activity directly to obtain cell lines expressing the heterodimer.

5 Still a further alternative is the development of a multicistronic vector encoding both subunits and one drug selection marker. Transfection of this vector and its amplification might more rapidly yield high expressing cell lines.

10 In any of the expression systems described above, the resulting cell lines can be further amplified by appropriate drug selection, resulting cell lines recloned and the level of expression assessed using the gamma interferon induction assay described herein.

Example 8. Biological Activities of Human NKSF

15 The following assays were performed using either the homogeneous NKSF described in Example 2 or a partially purified version of NKSF. The recombinant version of the molecule is expected to exhibit NKSF biological properties in these same assays or other
20 assays.

When fresh human peripheral blood mononuclear cells (PBMC) or phytohemagglutinin (PHA)-induced blasts are cultured with NKSF, significant amounts of gamma interferon are detected in the supernatant. Moreover,

NKSF synergizes with IL-2, phorbol dibutyrate (PdBu), and PHA in inducing gamma interferon production. Northern blot analyses show that NKSF, alone or in combination with other factors, induces accumulation of gamma
5 interferon mRNA. Gamma interferon message was found in both purified T and NK populations. Preincubation with the protein synthesis inhibitor, cycloheximide (CHX), leads to a superinduction of gamma interferon mRNA following stimulation with NKSF. HLA-DR(+) accessory
10 cells are required for gamma interferon production by T and NK cells. Induction of gamma interferon mRNA can be detected as early as 1 hour after treatment with NKSF of PHA blasts. The details of the assay are described below.

15 a. Gamma Interferon Induction Assay

NKSF activity was measured by the induction of gamma interferon (gamma-IFN) expression in cultures of human peripheral blood lymphocytes (PBLs). In the assay, 100 μ l of human PBLs suspended (10^7
20 cells/ml) in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS was added to 100 μ l of sample to be tested in a microtiter plate [U-bottom, 96-well, Costar, Cambridge, MA] and incubated for 18 hours at 37°C, 5% CO₂. Samples to be tested included purified
25 NKSF, dialyzed cell free supernatant from 48 hour phorbol

diester stimulated RPMI 8866 cells, and recombinant IL-2 [Genetics Institute, Inc., PCT application WO85/05124].

After incubation, 100 μ l of cell free supernatant was withdrawn from each well and the level of gamma-IFN

5 produced measured by radioimmunoassay [Centocor Gamma Interferon Radioimmunoassay, Centocor, Malvern, PA]. One unit of NKSF per ml is the concentration required to produce one-half of the maximal gamma-IFN produced in the presence of optimal concentrations of NKSF.

10 There was a linear positive correlation between the amount of gamma-IFN produced in each well to the amount of NKSF in culture.

In addition to gamma-IFN, NKSF induces T and NK cells to produce GM-CSF and tumor necrosis factor. The
15 assay of production of these cytokines is performed as above and the supernatant is assayed for the presence of the cytokines by specific biological assays or by radioimmunoassays [Cuturi et al, J. Exp. Med., 165:1581-1594 (1987)]. Alternatively, the induction of the
20 cytokine genes is measured by evaluating the accumulation of mRNA transcripts of the three cytokines in the lymphocytes treated with NKSF. Lymphocytes are cultured for 4 to 18 hours with NKSF, RNA is extracted by established methods, fractionated by agarose gel
25 electrophoresis, blotted on nitrocellulose, and hybridized with 32 P-labeled cDNA probes for the IFN-gamma,

GM-CSF, or tumor necrosis factor genes (Northern blotting). Extent of hybridization is determined by autoradiography and densitometry.

5 NKSF induces production of IFN-gamma and TNF
from purified human NK cells. When assayed as described
under the gamma interferon induction assay of part (a)
above, NK cells are able to lyse various target cells by
two mechanisms. One mechanism is spontaneous lysis, in
the absence of specific sensitization, of a variety of
10 target cells, including leukemia- and solid tumor-derived
cell lines, virus-infected cells, and, in some cases,
normal cell lines. The second mechanism is ADCC.
Preliminary evidence indicates that NKSF may enhance the
ability of NK cells to lyse more efficiently target cells
15 coated with IgG antibodies with an Fc portion able to
bind to the NK cell Fc receptor.

b. NK Assay

In order to assay for the enhancement of
NK cell spontaneous cytotoxicity by NKSF, PBLs or
20 purified NK cells (5×10^6 cells/ml) are incubated for 18
hours in RPMI 1640 medium, 10% heat inactivated FCS, in
the presence of various dilutions of NKSF. PBLs are then
washed and added, at PBL-target cells ratio from 1:1 to
100:1, to 10^4 ^{51}Cr -labeled target cells in a U-bottomed
25 microtiter plate (final volume 200 μl). After 4 hours,
the plates are centrifuged, the cell-free supernatant is

collected and lysis of target cells is evaluated by the release of the ^{51}Cr -label from the cells. NKSF increases several-fold the cytotoxicity of NK cells when assayed against the following target cells: malignant

5 hematopoietic cell lines (i.e. K562, Daudi, U937, HL-60, ML3, Molt 4, Jurkat, THP-1), solid tumor-derived cell line (rhabdomyosarcoma, melanoma), and normal foreskin-derived fibroblast strains. The enhancement of NK cell-mediated cytotoxicity by NKSF is not secondary to the

10 production of IFN-gamma, tumor necrosis factor, or IL-2, produced by the PBL treated with NKSF. The cytotoxic assay, the methods for NK cell purification, and for the quantitative evaluation of enhancement of NK cell-mediated enhancement by cytokines are described in detail

15 in G. Trinchieri et al, J. Exp. Med., 147:1314 (1978); G. Trinchieri et al, J. Exp. Med., 160:1147 (1984); and B. Perussia et al, Natural Immunity and Cell Growth Regulation, 6:171-188 (1987).

c. ADCC Assay

20 In a standard antibody dependent cell mediated cytotoxicity assay, preliminary results show that partially purified NKSF of the present invention enhanced NK cell killing of antibody coated tumor target cells in a dose dependent manner. For antibodies capable of

binding to the Fc receptor of the NK cell, the ADCC response of NK cells was enhanced by the addition of NKSF.

d. Co-mitogenic effect of NKSF

5 PBLs ($0.5 \times 10^6/\text{ml}$) are cultured in 200 μl of RPMI 1640 medium supplemented with 10% heat inactivated human AB serum. After 3 and 6 days the PBLs are pulsed for 6 hours with ^3H -thymidine and DNA synthesis (proliferation) is evaluated by the ^3H -thymidine uptake in
10 the cells by collecting the cells on glass filters using a Skatron cell harvester and counting the cell-associated ^3H -Thymidine by liquid scintillation using a Packard Tricarb beta counter. NKSF has minimal effect on PBL proliferation by itself, but is strongly co-mitogenic
15 with phytohemagglutinin (PHA-M Wellcome, 1:100) at day 6 of culture and with phorbol diesters (TPA or PDBu, 10^{-8} or 10^{-7}M , respectively) at both day 3 and day 6. Cell cycle analysis is performed by flow cytometry (Cytofluorograf 50H, Ortho Diagnostics) using a technique
20 combining DNA staining with immunofluorescence staining according to London et al, J. Immunol., 137:3845 (1986). This analysis has shown that the PBLs affected by the co-mitogenic effect of NKSF are T cells either CD4 or CD8 positive.

e. GM-CSF Induction Assay

Induction of GM-CSF expression in cultures of human PBLs was measured. In the assay, 100 μ l of human PBLs suspended (10^7 cells/ml) in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS was added to 100 μ l of sample to be tested in a microtiter plate [U-bottom, 96-well, Costar, Cambridge, MA] and incubated for 18 hours at 37°C, 5% CO₂. After incubation, 100 μ l of cell-free supernatant was withdrawn from each well and the level of GM-CSF produced measured by enzyme-linked immunosorbent assay (ELISA) using two murine monoclonal antibodies against human GM-CSF (3/8.20.5 and 2/3.1, supplied by Genetics Institute, Inc.) recognizing different epitopes. Using recombinant human GM-CSF (Genetics Institute, Inc.) as a standard, the detection limit of this assay was 50 pg/ml.

Numerous modifications and variations in practice of this invention are expected to occur to those skilled in the art.

CLAIMS

1. Natural killer cell stimulatory factor protein capable of inducing the production of gamma interferon in vitro in human peripheral blood lymphocytes and being substantially free from association with other proteinaceous materials.

2. The protein according to claim 1 comprising a subunit having an apparent molecular weight of approximately 40kD and having the same or substantially the same amino acid sequence of Table I.

3. The protein according to claim 1 comprising a subunit having an apparent molecular weight of approximately 30-35 kD and having the same or substantially the same amino acid sequence of Table II.

4. The protein according to claim 1 characterized biologically by a specific activity in a gamma interferon induction assay of greater than 1×10^7 dilution units/mg.

5. The protein according to claim 1 having one or more of the following characteristics:

- (1) an apparent molecular weight under non-reducing conditions on SDS PAGE of approximately 70-80 kD;
- (2) a subunit having an apparent molecular weight under reducing conditions on SDS PAGE of approximately 40 kD;
- (3) a subunit having an apparent molecular weight under reducing conditions on SDS PAGE of approximately 30-35 kD;
- (4) an isoelectric point of 4.3 on isoelectric focusing gel;
- (5) an isoelectric point of 4.8 on isoelectric focusing gel;
- (6) elution from hydroxylapatite column as a single peak;
- (7) elution from heparin-sepharose column as a single peak;
- (8) elution from an FPLC Mono-Q column as a single peak;
- (9) biological activity in a gamma IFN inducing assay with PBLs;
- (10) biological activity in a GM-CSF inducing assay with PBLs;

(11) biological activity in activating NK cells to kill leukemia and tumor-derived cells;

(12) biological activity in a tumor necrosis factor induction assay using PHA-activated T lymphocytes;

(13) co-mitogenic activity on peripheral blood T lymphocytes.

6. A process for preparing homogeneous NKSF comprising subjecting conditioned medium from RPMI 8866 to sequential purification through a QAE Zeta Prep cartridge, a lentil lectin column, a hydroxylapatite column, a heparin sepharose column and a fast protein liquid chromatography Mono-Q column, wherein said NKSF elutes from the latter column as a single peak.

7. The process according to claim 6 further comprising subjecting the Mono-Q column eluate to gel filtration chromatography.

8. The process according to claim 6 optionally including a reverse phase HPLC purification before said gel filtration chromatography.

9. A process for producing NKSF or a subunit thereof comprising culturing a cell line transformed with a DNA sequence encoding expression of NKSF or a subunit thereof in operative association with an expression control sequence therefor.

10. The process according to claim 9 wherein said DNA sequence comprises the same or substantially the same sequence of Table I, or a fragment thereof.

11. The process according to claim 9 wherein said DNA sequence comprises the same or substantially the same sequence of Table II, or a fragment thereof.

12. A DNA sequence coding for NKSF or a subunit thereof comprising a sequence of nucleotide bases the same or substantially the same as a sequence selected from the group consisting of:

- (a) the sequence of Table I;
 - (b) the sequence of Table II;
 - (c) fragments thereof; and
 - (d) sequences capable of hybridizing thereto.
-

13. A cell transformed with a DNA sequence of claim 12 in operative association with an expression control sequence.

14. The cell according to claim 13 comprising a mammalian or bacterial cell.

15. Homogeneous NKSF having a specific activity in the gamma interferon induction assay of greater than 1×10^7 dilution units per mg polypeptide.

16. A pharmaceutical composition comprising a therapeutically effective amount of NKSF or a subunit thereof in a pharmaceutically effective vehicle.

17. The composition according to claim 16 further comprising therapeutically effective amounts of an additional cytokine, hematopoietin, or growth factor.

18. The composition according to claim 16 where said cytokine is selected from the group consisting of IL-1, IL-2 and IL-6.

19. A plasmid vector comprising a DNA sequence of claim 12.

20. The plasmid vector according to claim 19 wherein the vector is pNK40-4.

21. The plasmid vector according to claim 19 wherein the vector is p35nksf14-1-1.

22. A method for treating cancer comprising administering to a patient an effective amount of NKSF or a subunit thereof.

23. The method according to claim 22 further comprising administering simultaneously or sequentially with said NKSF an effective amount of at least one hematopoietin, cytokine, growth factor or antibody capable of binding to the Fc portion of NK cells.

24. The method of claim 23 wherein said hematopoietin is IL-1, IL-2 or IL-6.

25. A method for treating infections comprising administering to a patient an effective amount of NKSF or a subunit thereof.

26. The method according to claim 25 wherein said infection is a viral or bacterial infection.

27. The method according to claim 26 wherein said infection is a nonresponsive viral infection.

28. The method according to claim 26 wherein said infection is AIDS.

29. Natural killer cell stimulatory factor protein substantially free from association with other proteinaceous materials comprising a first subunit having an apparent molecular weight of approximately 40 kD and having the same or substantially the same amino acid sequence of Table I, in association with a second subunit having an apparent molecular weight of approximately 30 kD and having the same or substantially the same amino acid sequence of Table II.

30. A method for treating infections comprising administering to a patient an effective amount of NKSF or a subunit thereof and IL-2 wherein a synergistic effect between NKSF and IL-2 is achieved.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/06332

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.C1.5	C 12 N 15/19	C 12 N 15/24
A 61 K 37/02	C 07 K 13/00	C 07 K 3/28
C 12 N 1/21		C 12 P 21/02
		C 12 N 5/16
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.C1.5	C 12 N A 61 K	C 12 P C 07 K
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
L, X	WO, A, 9005147 (GENETICS INSTITUTE, INC.) 17 May 1990, see the whole document (L document: throwing doubt on the priority claim) ---	1-24, 29
X	Proceedings of the National Academy of Sciences, volume 87, 5 September 1990, Immunology (Washington, US) A.S. Stern et al.: "Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells", pages 6808-6812, see page 6811; page 6812, line 36 - end --- -/-	1-5, 15- 18, 22- 24, 29
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
08-01-1992	- 6. 02. 92	
International Searching Authority	Signature of Authorized Officer	
EUR PEAN PATENT OFFICE	<div style="display: flex; align-items: center;"> <div style="margin-right: 20px;">Maria Peis</div> <div style="font-family: cursive; font-size: 1.2em;">Maria Peis</div> </div>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Journal of Experimental Medicine, volume 170, no. 3, 1st September 1989, (New York, US) M. Kobayashi et al.: "Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes", pages 827-845, see the whole article, especially page 829; page 836 and page 847, lines 7-14. ---	1-8,15- 18,22- 24,29
P,X	EP,A,0433827 (F. HOFFMANN-LA ROCHE AG) 26 June 1991, see the whole document, especially table I, figures 25 and 26; pages 32-35 and the claims ---	1-5,9- 24,29
P,X	The Journal of Immunology, volume 146, no. 9, May 1991 (Baltimore, US) S.F. Wolf et al.: "Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells", pages 3074-3081, see the whole article -----	1-26,29 -30

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 25-28 and 30 are directing to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compository.

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9106332

SA 52078

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/01/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9005147	17-05-90	AU-A- 4667389	28-05-90
		CA-A- 2002607	10-05-90
		EP-A- 0441900	21-08-91
EP-A- 0433827	26-06-91	AU-A- 6834990	27-06-91